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Selective inhibition of photosystem II in spinach by tobacco mosaic virus: an effect of the viral coat protein

Richard A.J. Hodgson, Roger N. Beachy and Himadri B. Pakrasi

Plant Biology Program, Department of Biology, Box 1137, Washington University, St. Louis, MO 63130, USA

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Leaves of *Spinacia oleracea* inoculated with tobacco mosaic virus (TMV) strain PV230 develop mild chlorotic and mosaic symptoms of infection. Thylakoid membranes isolated from these infected leaves showed a reduced F_v/F_m ratio for chlorophyll fluorescence kinetics, at 25°C. The photosystem II (PS II)-mediated electron-transport rate was inhibited 50%, whereas PS I activity was unaffected by virus infection. Protein analysis indicated that TMV coat protein was associated with thylakoids, in particular with the PS II fraction. The results demonstrate that TMV-infected *S. oleracea* shows inhibition of photosynthetic electron transport through PS II. We propose that the inhibition of photosynthetic activity results from the association of viral coat protein with the PS II complex.

Photosynthesis inhibition; Photosystem II; TMV; Viral coat protein; (*Spinacia oleracea*)

1. INTRODUCTION

Many stress conditions have their primary effects on photosynthesis and inhibit photosystem II (PS II) in particular [1]. Pathogens that invade plants and spread systemically impose a stress which may ultimately reduce photosynthesis [2]. Visible disease symptoms arising from viral pathogens can often include a mosaic pattern as well as leaf chlorosis and can be attributed to a deterioration of chloroplast structure, pigment composition and photosynthetic activity [2–4]. However, it has not been shown how, if at all, virus or virus component(s) induce damage to chloroplasts.

Evidence is accumulating for the hypothesis [5–7] that viral coat protein (CP) has a major influence on the induction and intensity of disease symptoms after viral infection. Transgenic plants expressing viral CP develop a protection to infection by the virus whose CP is incorporated into the host genome [8–10]. Mutagenesis studies show that the intensity of disease symptoms is changed when part of the CP or the entire CP cistron is deleted from the viral genome [11,12]. Recently, Reinero and Beachy [13] showed that CP from tobacco mosaic virus (TMV) is present in chloroplasts from infected tobacco plants. Coincident with infection with severe strains of TMV (PV230, PV39), which induce extensive yellow-white mosaic and chlorotic symptoms, was an accumulation of CP in chloroplasts [13,14] – first in the stroma and then in the thylakoid membranes. On the other hand, tobacco infected with a mild TMV strain (PV42) remains green because disease symptoms are masked. In PV42-infected plants there was little CP in chloroplasts [13]. There was also an indication that photosynthesis was differentially inhibited in plants infected with different viral strains [14].

The range of hosts for TMV is extensive [15] and may include spinach. Since the procedures for isolation and characterization of thylakoids and

Correspondence address: H.B. Pakrasi, Department of Biology, Box 1137, Washington University, St. Louis, MO 63130, USA

Abbreviations: PS, photosystem; CP, coat protein; TMV, tobacco mosaic virus; Chl, chlorophyll; MV, methyl viologen; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; DCPIP, 2,6-dichlorophenolindophenol; DMBQ, 2,5-dimethyl-*p*-benzoquinone

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subthylakoid fractions are well established for spinach, we decided to determine whether a severe strain of TMV, PV230, could systemically infect spinach in a manner similar to that described for tobacco. In this initial report we have examined whether CP is associated with a particular thylakoid fraction in infected leaves and whether photosynthetic electron transport is inhibited in TMV-infected spinach.

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L. cv. Bloomsdale Long Standing) was grown under greenhouse conditions supplemented with light during winter to maintain a 12 h day. During growth, plants were watered daily with a high N_2 nutrient solution. 2–3-week-old plants were inoculated with TMV [16] using 150 μl (0.5 μg/ml) of strain PV230 [17] or buffer (20 mM NaH_2PO_4, 1 mM EDTA; pH 7.8) for mock controls. Leaves were harvested 4 weeks after inoculation and the thylakoids isolated according to [18] except that the centrifugation steps were at $10000 \times g$ for 10 min. Kinetics of room temperature chlorophyll (Chl) fluorescence were assayed on a custom-built fluorometer as described [19]. For the fluorescence assay, thylakoids equivalent to about 5 μg Chl were resuspended in 3 ml buffer which contained 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA and 30 mM Hepes (pH 8.0). Fluorescence transients were detected at 690 nm ($\pm$ 10 nm) after excitation with 440 nm ($\pm$ 20 nm) light. Assays were repeated, after a 3 min dark adaption period, at least four times on each sample. The F_0 level was determined from a fast scan of the initial 100 ms illumination. The F_m level was evaluated from a separate transient after about 5 s. Electron-transport rates were assessed as rates of O_2 evolution or uptake on a YSI (Yellow Springs Instruments, OH) O_2 electrode. The reaction buffer was the same as that used for fluorescence analysis and contained thylakoids equivalent to 30 μg Chl with either: (i) 1 mM methyl viologen (MV) plus 2.5 mM NH_4Cl for PS II + PS I activity; (ii) 8 μM DCMU, 100 μM DCPIP, 1 mM ascorbate, 1 mM MV plus 2.5 mM NH_4Cl for PS I activity; or (iii) 0.75 mM DMBQ plus 1 mM $K_3Fe(CN)_6$ for PS II activity. Thylakoid pigments were determined according to [20,21] after extraction in 80% acetone. PS II and PS I particles were isolated as in [22,23] respectively. Lithium dodecyl sulfate (LDS)-PAGE of samples was performed on 12.5–20% gradients of acrylamide as described [24]. Western immunoblot analysis of the gel was performed using antibodies (raised in rabbits) against CP from U_1, the common TMV strain [13] and goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad, USA) using the procedure recommended by the suppliers.

3. RESULTS

Spinach inoculated with TMV PV230 started to show mild yellowing and mosaic symptoms of infection after 2 weeks. Analyses of electron transport and pigment composition in thylakoids from infected leaves are shown in fig.1. The Chl fluorescence transients from thylakoids of infected leaves showed a reduced F_v/F_m ratio which was equivalent to that observed for control thylakoids in the presence of DCMU. However, the slope of increase in fluorescence from F_0 to F_m (i.e. F_v) was much less in the virus-infected vs DMCU-treated sample (fig.1). Corresponding to the reduction in F_v/F_m, for PV230 samples, was an inhibition of O_2 exchange in thylakoids (fig.1). There was 35% inhibition of whole-chain electron transport which was the result of specific inhibition, of about 50%, in PS II activity. PS I electron transport was not inhibited in these thylakoids.

Analysis of thylakoid pigments (fig.1) indicated that infection with TMV resulted in a slightly decreased Chl *a*/*b* ratio but had no effect on the ratio of Chl/carotenoids.

The protein profiles of thylakoids, and PS II and PS I particles, as analysed on LDS-PAGE, are shown in fig.2A and a Western immunoblot of an equivalent gel is presented in fig.2B. Thylakoids from infected leaves contained a new band at about 20 kDa which comigrated with CP. Purified CP has an estimated molecular mass of 17.5 kDa and

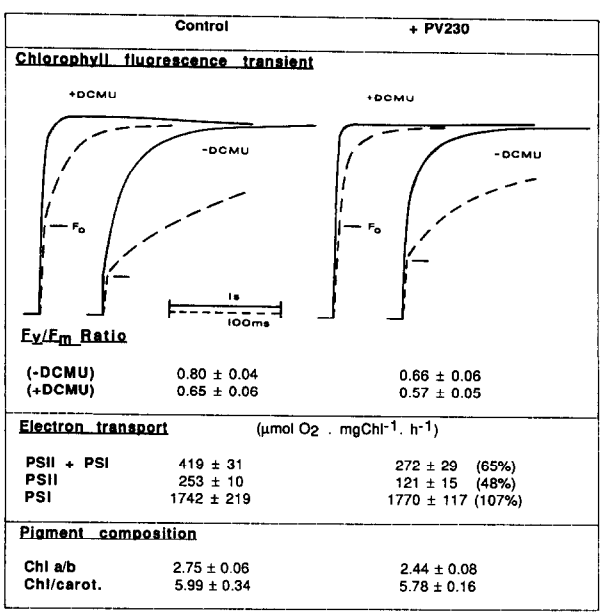

Fig.1. Kinetics of chlorophyll fluorescence, rates of electron transport and pigment composition of thylakoids from spinach infected for 28 days with TMV strain PV230. The fluorescence transients in the presence of DCMU have been normalized to equivalent F_m. All values are means $\pm$ SD ($n > 4$).

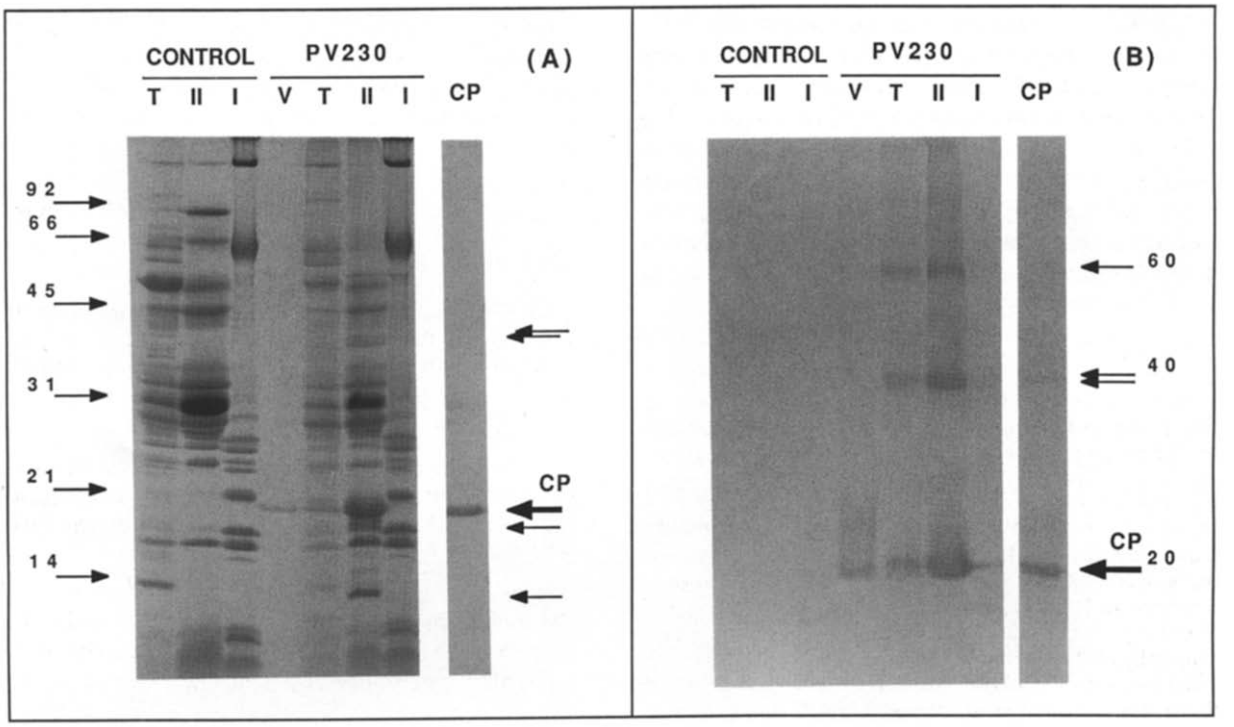

Fig.2. (A) LDS-PAGE and (B) Western immunoblotting of polypeptides from thylakoids (T), PS II particles (II) and PS I particles (I) of spinach infected for 28 days with TMV strain PV230. LDS-solubilized PV230 virus (V) and coat protein from TMV strain U_1 (common strain) (CP) have been run on the same gel as standards. In (A), the positions of new bands (including CP) are shown on the right and molecular mass markers (kDa) on the left. In (B), immunoreactive bands are indicated on the right.

migrates on acrylamide gels at about 20 kDa [8,12]. The CP appeared to be concentrated in PS II preparations and was not visible as a stained band in the PS I fraction (fig.2A). In addition to the CP band, PS II preparations had several other new bands at about 14, 18 and 40 kDa. The identities of these proteins are currently unknown. There was also an absence of three high molecular mass bands. The immunoblot showed proteins, in thylakoid samples, of about 20, 40 and 60 kDa that were recognized by the αCP antibodies (fig.2B). The purified CP showed bands at 20 and 40 kDa. Similar high molecular mass bands (40 and 60 kDa) have been observed in tobacco thylakoids and have been interpreted as being due to the presence of oligomers of CP [13]. The immunoblot also revealed that the PS I particles contained a small amount of CP. None of the mock controls showed any CP-related band.

The initial supernatant from PV230 tissue homogenate (obtained after the first centrifugation step during the isolation of thylakoids) was added to thylakoids from mock infected leaves to determine whether the free virus or CP that was in solution could become bound to thylakoids. The treatment had no effect on the F_v/F_m ratio and no CP band was present in these thylakoid samples (not shown).

4. DISCUSSION

Our results demonstrate that infection of spinach with TMV is associated with accumulation of TMV-CP at the PS II complex in thylakoids and a corresponding specific inhibition of electron transport mediated by the PS II complex. The reduced F_v/F_m ratio for Chl fluorescence in PV230-infected plants (fig.1) is indicative of inhibition of electron transport via PS II. This observation is supported by the severe inhibition of rates of PS II-mediated electron transport (fig.1). Interestingly, the capacity for electron transport through PS I is not affected by virus infection. This suggests that tissues showing symptoms of infec-

tion may perform cyclic phosphorylation and ATP synthesis in preference to whole-chain electron transport and reduction of $NADP^+$. The effect of viral infection on photosynthesis has been widely examined and it is noted that inhibition of electron transport is almost totally a function of inhibition at PS II and not PS I [3,4]. Thus, it would appear that some factor associated with the viral infection promotes inhibition at a specific location (PS II) in the chloroplast.

Protein analysis, by LDS-PAGE and Western immunoblotting, of thylakoids, and PS II and PS I particles revealed that TMV-CP was abundant in the thylakoid fraction of PV230-infected leaves. The CP was principally associated with PS II and since this site shows inhibition of electron transport (fig.1), the association of CP with PS II may cause the inhibition of PS II activity. The interaction between CP and PS II probably does not involve extrinsic binding of CP to the thylakoid, for example, at the Q_B (herbicide-binding) site. Herbicides like DCMU inhibit electron transport and induce a rapid rise in Chl fluorescence from F_0 to F_m (fig.1) which is not observed in thylakoids from PV230-infected tissue. Furthermore, Reinero and Beachy [13] showed that protease treatment of tobacco thylakoids did not remove the associated CP. Thus, it is unlikely that the association of CP with thylakoid membranes is due to loose external interactions. The nature of the binding of CP and its location in PS II are currently being investigated in our laboratory.

Since TMV infection of spinach promotes association of CP with the PS II complex and this correlates with inhibition of electron transport through PS II, TMV-infected samples may provide further insight into the relationship between structural organization and function in PS II. In addition, the novel way in which CP has a directed incorporation into thylakoids at PS II warrants closer examination to determine what attribute of the CP governs this targeting. Finally, from a phytopathology viewpoint, it will be important to determine the role that this CP-induced inhibition of electron transport plays in virus disease development.

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